

## REMARKS

Reconsideration of this application is respectfully requested in view of the foregoing amendments and remarks contained herein. This application pertains to a new potassium channel TWIK-1. Specifically, it relates to an isolated and purified TWIK-1 protein which constitutes a potassium channel having two pore domains and four transmembrane domains.

The Office Action contains provisional rejections under obviousness type double patenting of Claims 11 and 12 over 5 - 13 of co-pending Application No. 09/436,265, over Claims 9 - 15 of co-pending Application No. 09/939,483, and over claims 9 - 15 of co-pending Application Nos. 09/939,484 and 09/892,360. As none of these applications have issued, we ask that these provisional-type double patenting rejections be held in abeyance until at least one of the foregoing claims issues, in which case the matter may be taken up again.

Claims 11 and 12 have been rejected under 35 U.S.C. §101 as lacking a credible, substantial, specific, or well established utility. The protein claimed in Claim 11 has a fundamental role in the transport of potassium in a large number of cells. A deficiency in the protein translates to the inability of a cell to properly transport potassium. Thus, the protein itself has a critical role in cell biology. The protein can, thus, be used to screen for the deficiency of the TWIK-1 protein in tissues, indicating a diseased state or a propensity to develop such a diseased state. Specifically, one method of doing this, which is within the skill of one skilled in the art, is to use the potassium channel protein as an immunogen to make an antibody. The labeled antibody can then be used to identify cells containing the protein and, thus, to also identify those cells deficient in the protein. The cells are then useful as a tool for testing treatments of cells deficient in this protein. Applicants believe this is a credible, substantial, specific, and well established utility and accordingly request that the

rejection under 35 U.S.C. §101 be withdrawn. The Applicants also enclose an article entitled *Wildemann et al.*, 2001, Mol. Med., Vol. 7, pages 193 – 199. This article discloses the expression of TWIK-1 (=HOHO1) in nervous system pathology. This article reinforces the demonstrated utility of TWIK-1. References 36 and 37 of the article prove that HOHO1 corresponds to the TWIK-1 channel of this invention.

Claims 11 and 12 have been rejected under 35 U.S.C. §112 as not supported by either a credible asserted utility or a well established utility. In light of the arguments set forth above, Applicants contend that the utility of the invention of Claims 11 and 12 is well established and respectfully request that this rejection be withdrawn along with the rejection under 35 U.S.C. §101.

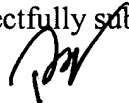
Claims 11 and 12 have also been "rejected under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the Specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." This rejection relates to the claiming of a genus of TWIK proteins having two pore domains and four transmembrane domains, or functionally equivalent derivatives. Even absent any guidance in the Specification, Applicants believe that one skilled in the art would be able to make conservative amino acid substitutions to the protein set forth in SEQ ID NO:2, to produce a functional protein similar to, yet having one or two amino acids different from, that set forth in SEQ ID NO:2. Thus, once given SEQ ID NO:2, one skilled in the art would be able to produce, test, and confirm a series of very close yet not exactly identical proteins using conservative amino acid substitutions (e.g. substitutions of isoleucine or leucine for valine). Such obvious variants should not go unclaimed simply because such basic biochemistry may not be explicitly set forth in the Specification. Other basic chemistry, such as the method for building a

protein one amino acid at a time, is similarly not set forth in the Specification. However, the absence of such basic teaching in no way compromises the ability of one skilled in the art to make and use the invention as claimed.

Furthermore, the specification sets forth methods of identifying other proteins which fall within the scope of Claim 1. Attention is directed to page 7 of the Specification where primary structural features of TWIK-1 are set forth. The protein has two P domains. Other than the P domains, no significant alignment was seen between TWIK-1 and a  $k^+$  channel cloned in yeast which also had two P domains. This indicates that the P domains are highly conserved, while the remainder of the molecule may not be. Analysis of the hydrophobicity of such a potassium channel protein will reveal the presence of transmembranal domains. Furthermore, the Specification notes that a simple comparison of the complete sequence of TWIK-1 with the sequences of the Genbank database allowed identification of at least five genes of *Caenorhabditis elegans*, which had been characterized in the context of the nematode sequencing project, which potentially code for structural homologs of TWIK-1. Therefore, homologs can be identified through such a simple procedure, expressed, and tested. Thus, the Specification provides more than adequate support to allow one skilled in the art to identify and isolate or produce other potassium channel proteins having two P domains and four transmembrane domains. Accordingly, Applicants respectfully request that this rejection be withdrawn.

With these amendments and remarks, Applicants believe that all claims as currently drafted are clearly allowable as proper claims and favorable notification to that effect.

Respectfully submitted,



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**In the Title** (Clean Copy)

Please amend the title to read:

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ISOLATED TWIK-1 POTASSIUM CHANNEL PROTEINS

**In The Specification** (Clean Copy)

Please add the following as the first line of the specification:

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This application claims benefit of U.S. Patent Application Serial No. 08/749,816, filed 15

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November 1996, now U.S. Patent No. 6,013,470.

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**In the Abstract** (Clean Copy)

Please substitute the following for the abstract in this case:

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Abstract Of The Disclosure

3 This invention relates to the cloning of a member of a new potassium channel named TWIK-1. More specifically, it relates to an isolated and purified nucleic acid molecule coding for a protein constituting a potassium channel exhibiting the proper-ties and structure of the TWIK-1 type channel, as well as the protein coded by this nucleic acid molecule. The invention also relates to the use of this nucleic acid molecule to transform cells, and the use of these cells expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel for the screening of drugs.

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**In the Claims** (Clean Copy)

Please amend the claims as shown:

1. (As filed) Isolated and purified nucleic acid molecule coding for a protein constituting a protein channel exhibiting the properties and structure of the TWIK-1 type channel.
2. (As filed) Isolated and purified nucleic acid molecule coding for a protein constituting a potassium channel, characterized in that it codes for the protein the amino acid sequence of which is represented in the attached sequence list as number SEQ ID NO:2 or a functionally equivalent derivative of this sequence.
3. (As filed) Nucleic acid molecule according to claim 2, the sequence of which is represented in the attached sequence list as number SEQ ID NO:1.
4. (As filed) Vector containing a nucleic acid molecule according to one of claims 1 to 3.
5. (As filed) Procedure for the production of a protein constituting a potassium channel exhibiting the properties and structure of the TWIK-1 type channel consisting of:
  - transferring a nucleic acid molecule according to one of claims 1 to 3 or a vector according to claim 4, into a cellular host,
  - culturing the cellular host obtained in the preceding step under conditions allowing the production of potassium channels exhibiting the properties of TWIK-1,
  - isolating, by any suitable means, the proteins constituting the potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

6. (As filed) Procedure for the expression of a potassium channel exhibiting the properties and structure of the TWIK-1 type channel consisting of:

- transferring a nucleic acid molecule according to one of claims 1 to 3 or a vector according to claim 4, into a cellular host,

- culturing the cellular host obtained in the preceding step under conditions allowing the expression of potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

7. (As filed) Procedure according to one of claims 5 or 6, characterized in that the cellular host is selected from among the prokaryotes or the eukaryotes and, particularly, from among the bacteria, the yeasts, mammal cells, plant cells or insect cells.

8. (As filed) Cell expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel obtained by the procedure according to claim 6 or 7.

9. (As filed) Procedure for screening substances capable of modulating the activity of the potassium channels of the TWIK-1 type channel, characterized in that:

- one brings into contact variable amounts of a substance to be tested with the cells expressing the potassium channels exhibiting the properties and structure of the TWIK-1 type channel according to claim 8, then

- one measures, by any suitable means, the possible effects of said substance on the currents of the potassium channels exhibiting the properties and structure of the TWIK-1 type channel.

10. (As filed) Pharmaceutical composition for the compensation of a deficiency in the potassium channels at the level of one or more tissues, characterized in that it comprises nucleic acid molecules according to one of claims 1 to 3, or a vector according to claim 4, or cells according to claim 8.

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11. (Twice Amended) An isolated and purified TWIK-1 protein constituting a potassium channel having two pore domains and four transmembrane domains.

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12. (Previously amended) The protein according to claim 11 comprising SEQ ID NO:2, or a functionally equivalent derivative of said sequence.

13. Previously canceled.

14. (As filed) Monoclonal or polyclonal antibody directed against a protein according to claim 11 or 12.

15. (As filed) A method of screening for a substance capable of modulating the activity of a mammalian protein comprising 2 P domains and 4 transmembrane segments, which protein is competent to transport potassium across a membrane, comprising contacting pre-selected amounts of the substance to be tested with cells expressing the potassium transport channel, measuring the effects of said substance on the potassium transport activity of the protein, and identifying the substance that has a positive or negative effect on said transport activity.

16. (As filed) The method of claim 15 wherein the protein which is competent to transport potassium across a membrane is human.

17. (As filed) The method of claim 15 wherein the cell expressing the potassium transport protein is transformed with a self replicating vector comprising a nucleic acid sequence encoding a mammalian protein comprising 2 P domains and 4 transmembrane segments, which protein is competent to transport potassium across a membrane.

18. (As filed) The method of claim 17 wherein the self replicating vector comprises a nucleic acid sequence encoding a human potassium transport protein.

19. (As filed) The method of claim 18 wherein the self replicating vector comprises SEQ ID No:1.

20. (As filed) A substance, identified by the method of claim 15, which is capable of positively or negatively influencing the transport activity of a potassium transport channel.

21. (As filed) The substance of claim 20 which influences the transport activity of the potassium transport channel comprising 2 P domains and 4 transmembrane segments.

22. (As filed) The substance of claim 21 which influences the transport activity of the potassium transport channel represented by SEQ ID No:2.

23. (As filed) A pharmaceutical composition for the treatment of diseases caused by the malfunction of a potassium transport channel, comprising the substance of claim 20.

24. (As filed) The pharmaceutical composition of claim 23 which influences the transport activity of a potassium transport channel comprising 2 P domains and 4 transmembrane segments.

25. (As filed) The pharmaceutical composition of claim 24 which influences the transport activity of the potassium transport channel represented by SEQ ID No:2.

26. (As filed) The pharmaceutical composition of claim 23 which is useful for the treatment of diseases selected from the group consisting of epilepsy, heart arrhythmias, vascular diseases, neurodegenerative diseases, ischemia or anoxia, endocrine diseases associated with anomalies of hormone secretion, and muscle diseases.

## Identification by mRNA Differential Display of Two Up-regulated Genes as Candidate Mediators of AIDS Dementia

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Contributed by S. Snyder. Accepted November 20, 2000

### Abstract

**Background:** In the dementia associated with acquired immunodeficiency syndrome (AIDS), indirect pathomechanisms are important mediators of progressive neuronal injury and variable candidate molecules of potential pathogenetic importance have been identified. **Materials and Methods:** In an attempt to characterize additional mediators of human immunodeficiency virus type 1 (HIV-1)-induced neurotoxicity *in vivo* we have adapted the mRNA differential display technique to monitor the gene expression pattern in postmortem cortical tissue from AIDS patients with ( $n = 7$ ) and without ( $n = 8$ ) cognitive impairment as well as from HIV-1 seronegative controls ( $n = 4$ ). **Results:** Out of 29 differentially expressed cDNAs, two cDNA clones had confirmed variation of transcriptional regulation as assessed by reverse Northern analysis and

gene-specific reverse transcription polymerase chain reaction (RT-PCR) and were up-regulated in the cortex of patients with AIDS dementia. Nucleotide sequence analysis of the two cDNAs identified known genes not previously associated with the pathogenesis of AIDS dementia, including the neurotrophin receptor tyrosine kinase receptor B (TrkB) and the potassium channel human open rectifier  $K^+$  channel (ORK) homologous open reading frame (HOKO1).

**Conclusions:** The altered expression of these transcripts may contribute to AIDS dementia through the enhancement of microglial activation and immunologic nitric oxide synthase (iNOS) activity by abnormal neurotrophic regulation and interference with membrane excitability through disturbance of local ion homeostasis.

### Introduction

The pathogenesis of the dementia associated with the acquired immunodeficiency syndrome (AIDS) is incompletely elucidated. The human immunodeficiency virus type 1 (HIV-1) mediates a productive infection of brain macrophages and microglia. Direct infection of neural cell types does not occur, although there is a drop in neuronal density as well as synaptic loss and dendritic simplification (1-6). The mechanisms involved in these neuronal changes appear to be indirect and multifactorial. The available evidence suggests that the release of various proinflammatory and neurotoxic molecules from activated macrophages and microglial cells are major determinants of brain damage (7-10). In most studies,

mediators of potential importance in the pathogenesis of AIDS dementia, such as cytokines, chemokines, metalloproteinases, excitotoxic transmitters, and the neurotoxic effects of nitric oxide (NO) were studied selectively. Here, we have monitored the gene expression pattern in postmortem brain tissue obtained from HIV-1 infected patients with and without dementia as well as from normal controls. Using reverse transcription polymerase chain reaction (RT-PCR)-based mRNA differential display (11-13) we attempted to characterize candidate genes not previously associated with the HIV-1-induced disease process in the brain.

### Material and Methods

#### Patients

We tested tissue from a total of 19 patients. Fifteen patients were HIV-1 seropositive and diagnosed with AIDS before death, and four individuals were HIV-1 seronegative. Of the 15 patients with AIDS, 8 had no cognitive impairment and 7 had dementia.

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Dementia was categorized according to the Memorial Sloan Kettering (MSK) criteria (14,15) and was mild (MSK 1 or 2) in three cases and severe (MSK 3 or 4) in four cases. Tissue was collected at autopsy, rapidly frozen in isopentane, and stored at  $-70^{\circ}\text{C}$ . Cortical specimens were obtained from the frontal lobe in all cases. The presence of central nervous system (CNS) opportunistic infections or lymphoma was excluded by neuroimaging, cerebrospinal fluid analysis, and postmortem histopathologic evaluation of brain tissue sections. HIV-1 seronegative control specimens were obtained from patients without CNS lesions. The causes of death in control patients included pneumonia ( $n = 1$ ) and trauma ( $n = 3$ ). Postmortem delays in tissue collection were  $8.75 \pm 2.75$  hr for HIV-1 seronegative controls and  $15.06 \pm 8.44$  hr for patients with AIDS. CD4 counts (number of cells per cubic millimeter) of HIV-1 positive individuals were  $107.83 \pm 89.53$  (no dementia),  $80.0 \pm 60.81$  (mild dementia), and  $33.0 \pm 60.75$  (severe dementia).

#### Differential Display mRNA Analysis

Differential display RT-PCR was carried out using tissue obtained from AIDS patients with severe dementia, no dementia, and normal controls. Total cellular RNA was extracted from frozen brain tissue using RNeasy (AGS, Heidelberg, Germany) according to the manufacturer's instructions. RNA was dissolved in 50  $\mu\text{l}$  diethyl pyrocarbonate-treated water and incubated for 10 min at  $37^{\circ}\text{C}$  and for 5 min at  $95^{\circ}\text{C}$  with 10 U ribonuclease-free deoxyribonuclease I (Boehringer, Mannheim, Germany) to remove chromosomal DNA contamination. Reverse transcription of 2  $\mu\text{g}$  total RNA was performed with Superscript II reverse transcriptase (Gibco BRL, Karlsruhe, Germany) using one of three different one base-anchored oligo-dT primers to subdivide mRNA into three subpopulations (16). Control reactions were performed in the absence of reverse transcriptase. One hundred nanograms of the cDNAs were then amplified in a 20- $\mu\text{l}$  reaction in the presence of the respective 3'-anchored oligo-dT primer (2.5  $\mu\text{M}$ ), 1 out of 29 different 10-mer 5'-arbitrary primers (500 nM),  $\text{MgCl}_2$  (1.625  $\mu\text{M}$ ), dNTPs (8  $\mu\text{M}$ ), and 1 U Taq polymerase (AGS). In control reactions, water was substituted for cDNA. Thermal cycling conditions were as follows:  $94^{\circ}\text{C}$  (30 sec),  $42^{\circ}\text{C}$  (60 sec),  $72^{\circ}\text{C}$  (30 sec) for 40 cycles and a final extension step at  $72^{\circ}\text{C}$  for 5 min. PCR products were loaded on a 12.5% polyacrylamide gel (Pharmacia, Freiburg, Germany) and detected by silver staining. Differentially regulated amplification products were defined as those bands that were either present or absent in cDNA subpopulations derived from AIDS patients with severe dementia as compared to nondemented AIDS patients or normal controls. Further characterization of selected bands demonstrating differential expression was carried out if the amplification pattern was reproduced with a different

cDNA preparation in a second PCR reaction. PCR products of interest were cut out from the gel, eluted with 20  $\mu\text{l}$  sterile water, and reamplified by PCR in a 50  $\mu\text{l}$  reaction using the appropriate primer set. The reaction mixture contained 2 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  dNTP, 1.25  $\mu\text{M}$  3'-anchored oligo-dT primer, 250 nM 5'-arbitrary primers, and 1 U AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany). Reamplification PCR proceeded over 45 cycles. Cycling conditions were as described for differential display RT-PCR except on initial denaturation step at  $95^{\circ}\text{C}$  for 8 min. Reamplified cDNA fragments were subcloned into the plasmid vector pCR II using the pTA Cloning Kit (Invitrogen, Groningen, Netherlands). Inserted cDNAs of correct size were used for DNA size blot analysis and nucleotide sequence assessment.

#### DNA Slot Blot Analysis (Reverse Northern Analysis)

Synthesized cDNA fragments were heat denatured and approximately 5  $\mu\text{g}$  was applied in duplicate to a nylon membrane with the use of a Minifold 1-Spot Blotter (Schleicher & Schuell, Dassel, Germany). Following baking for 1 hr at  $80^{\circ}\text{C}$  in a vacuum oven, membranes were equilibrated in  $5\times$  SSC, 50% formamide, 1% SDS, and 5% blocking powder. For reverse Northern hybridization digoxigenin (dig)-11-dUTP-labeled cDNA was generated with a dig-cDNA Synthesis Kit (Boehringer Mannheim) from 5  $\mu\text{g}$  of RNA preparations derived from cortical tissue specimens adjacent to those used for mRNA differential display analysis. Heat-denatured cDNA prepared from cortical tissue of nondemented and severely demented AIDS patients and from normal controls was added separately to the hybridization solutions containing one of the duplicate dot blotted membranes. Hybridization was carried out for 16 hr at  $42^{\circ}\text{C}$ . Membranes were washed twice in  $0.1\times$  SSC, 0.1% SDS at  $68^{\circ}\text{C}$ . Colorimetric detection was performed with a Dig-Detection Kit (Boehringer Mannheim).

#### Nucleotide Sequence Determination

Both reamplified fragments and subcloned inserts with confirmed differential expression as determined by reverse Northern analysis were purified using QIAquick spin columns (Qiagen, Düsseldorf, Germany). Sequencing was carried out by the fluorescent dideoxy chain termination method utilizing an automated DNA sequencer (ABI Prism 310, Applied Biosystems). The nucleotide sequences obtained were compared with known sequences by searching the GenBank and EMBL databases with BLAST algorithms.

#### Quantitative RT-PCR

Differential gene expression was further confirmed by SYBR Green real-time quantitative PCR and total RNA derived from brain tissue of all patient groups was tested. Two micrograms of the RNA preparation, which had also been used for the reverse Northern analysis, was reverse transcribed with

random hexamers using the GeneAmp RNA Core Kit (Applied Biosystems). Primers for the sequences of interest were designed with the Primer Express software (Applied Biosystems). Detection of  $\beta$ -actin mRNA was used to normalize the expression of target mRNAs.  $\beta$ -Actin specific primers were provided by Applied Biosystems. Coamplification of  $\beta$ -actin cDNA and target cDNAs were carried out in 3-fold parallel reactions using SYBR Green reagents (Applied Biosystems). A negative control was included in each reaction set. Reaction mixtures contained 200 ng cDNA, 3 mM  $MgCl_2$ , 250  $\mu$ g dNTPs, 300 nM of each primer, 1.25 U AmpliTaq Gold<sup>TM</sup>, and 3 U AmpErase in a total volume of 30  $\mu$ l. Thermal cycling proceeded in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) with 95°C (10 min) followed for 40 cycles with 60°C (1 min), and 95°C (19 sec). The quantity of target cDNAs was calculated with the use of the AACr method as described in the GeneAmp 5700 Sequence Detection System user's manual (Applied Biosystems). Results are defined as the target gene expression normalized against the  $\beta$ -actin gene expression in cortical tissue from patients with severely, mildly, and nondemented AIDS patients relative to the respective gene expression in cortical tissue from normal controls.

#### Statistical Analysis

The expression of selected genes as assessed by quantitative RT-PCR was analyzed by using Fisher's least significance difference test.

## Results

### Differential Display mRNA Analysis

We performed differential display RT-PCR with a total of 77 primer combinations. Twenty-nine amplification reactions were carried out with the one-base anchored T11A oligo-dT primer; 25 and 23 reactions were performed with one-base anchored T11C or T11G oligo-dT primers, respectively. We identified 29 PCR products that were differentially expressed between brain tissues obtained from severely demented and nondemented AIDS patients or normal controls. Figure 1 shows one representative PCR product that was reproducibly present in cortical samples from AIDS patients with severe dementia and less prominent in the cortex of two AIDS patients as compared to cortical specimens from normal controls.

### Reverse Northern Analysis

Reverse Northern analysis was performed to confirm the gene regulation patterns observed in the mRNA differential display study. From the 29 differentially expressed PCR products, 25 bands were successfully reamplified and screened by direct nucleotide sequence analysis. Eight fragments turned out to be artifacts and were not further assessed. The remaining 17 fragments were subcloned to be used as probes in

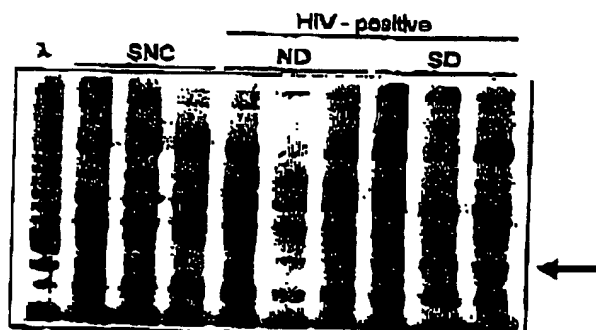


Fig. 1. Differential display RT-PCR comparing mRNAs extracted from cortical tissue obtained at autopsy from HIV-1 seronegative controls (SNC, lanes 2 through 4) and from HIV-1 infected patients with no dementia (ND, lanes 5 through 7) or severe dementia (MSK 3 or 4) (SD, lanes 8 through 10).  $\lambda$  is the DNA length standard. PCR products were loaded on a 12.5% polyacrylamide gel and detected by silver staining. The illustrated primer combination identified one distinct fragment, which was up-regulated in the cortex of three demented and two nondemented HIV-1 infected subjects (arrow). Primers included T11G as 3' anchor primer and 3'-AAGCTTACGG TACAC-3' as random primer.

DNA slot blots prepared with cDNAs from cortical tissues of nondemented and severely demented AIDS patients and from normal controls. Three of the 17 subcloned PCR products yielded hybridization signals that reproduced the expression patterns obtained by mRNA differential display (Fig. 2). The remaining 12 reamplified and subcloned PCR fragments generated discordant hybridization patterns and were not studied further.

### Sequence Homology

Sequence analysis of the three cloned cDNA fragments with confirmed differential expression revealed

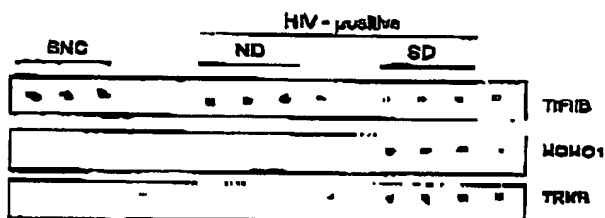


Fig. 2. Reverse Northern analysis confirming alternate gene regulation patterns for three differential display bands identified as TIF1B, HMO1, and TRIM by nucleotide sequence analysis. DNA slot blots prepared with the three fragments were probed with cDNAs derived from the cortex of SNC and from HIV-1 infected patients with ND or SD (MSK 3 or 4). Consistent with differential display RT-PCR reverse Northern analysis demonstrates down-regulation of TIF1B and up-regulation of HMO1 and TRIM in brain tissue samples from AIDS patients with SD as compared to AIDS patients with ND and SNCs.

homologies with three distinct genes. The three gene fragments were highly homologous to tyrosine kinase receptor B (TrkB), the potassium channel coding gene human open rectifier K<sup>+</sup> channel (ORK)-homologous open reading frame (HOHO1), and the general transcription initiation factor IIB (TIFIB).

#### Quantitative RT-PCR

The mRNA expression of the three genes identified by nucleotide sequencing was assessed by quantitative SYBR Green real-time PCR using primers with specificity for human TrkB, HOHO1, and TIFIB. Total RNA derived from cortical tissue specimens of all patient groups was included in the analysis. Results are outlined in Fig. 3. There was a 41-fold increase in TrkB mRNA in the cortex of severely demented AIDS patients as compared to the cortex of HIV-1 seronegative persons. TrkB mRNA expression was also increased in brain tissue of AIDS patients without or with mild cognitive impairment, although it was less pronounced (2 fold and 23 fold, respectively). HOHO1 gene transcripts were moderately elevated in cortical tissue of all patients with AIDS as compared to HIV-1 seronegative controls. Increases were 6.1-fold (nondemented AIDS patients), 5.7-fold (mildly demented AIDS patients), and 9.4-fold (severely demented AIDS patients). Quantitative RT-PCR of TIFIB mRNA was discordant with the results of mRNA differential display and reverse Northern analysis, and did not confirm the down-regulation of transcript levels. The amount of TIFIB was similar in the cortex of AIDS patients with severe cognitive dysfunction as compared to the control group (1.07-fold) and mildly down-regulated in the remaining patients (0.66-fold and 0.5-fold in AIDS patients without or with mild cognitive impairment).

#### Discussion

Using the mRNA differential display technique and 77 primer combinations, we identified 29 cDNAs that reproducibly showed different expression patterns between brain tissues from AIDS patients with severe dementia and nondemented AIDS patients or HIV-1 seronegative persons. Differing transcriptional regulation was confirmed for three cDNAs by reverse Northern analysis. We identified from these three bands three known genes, the tyrosine kinase receptor TrkB for the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5), the potassium channel HOHO1, and the general transcription initiation factor TIFIB. Up-regulation of TrkB and HOHO1 in the cortex of severely demented AIDS patients and less pronounced in the brain of nondemented and mildly demented AIDS patients was reproduced by quantitative RT-PCR with selected primers. Quantitative RT-PCR, however, did not

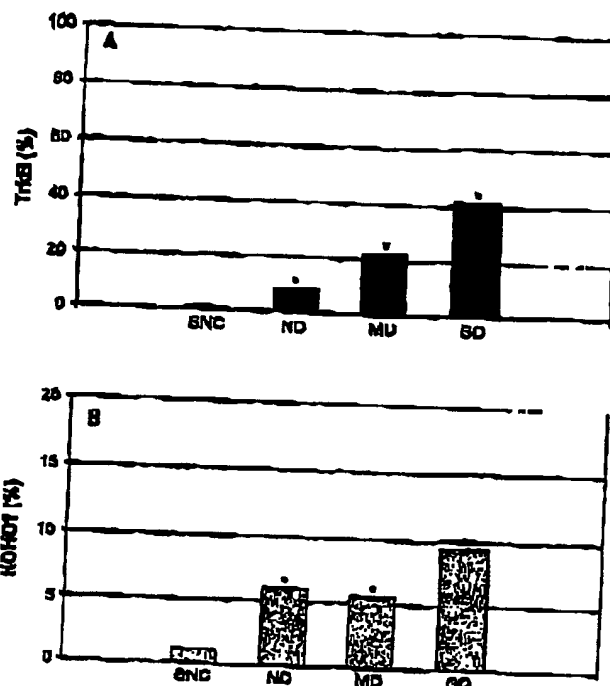


Fig. 3. Quantitative SYBR Green real-time PCR with TrkB and HOHO1 specific primers. Amplification was carried out using cortical tissue obtained at autopsy from SMCs ( $n = 4$ ) and from AIDS patients with ND ( $n = 3$ ), mild dementia (MD,  $n = 3$ ), and SD ( $n = 4$ ). TrkB and HOHO1 mRNA levels were normalized against  $\beta$ -actin mRNA levels and their expression in the cortex of AIDS patients with ND, MD, and SD is illustrated relative to the cortex of SMCs. (A) Expression of TrkB mRNA. TrkB mRNA levels are 41-fold increased in the cortex from AIDS patients with SD as compared to brain tissue of SMCs ( $p = .001$ , Fisher's least significance difference test). TrkB transcription is 23 fold and 2 fold up-regulated in the brains of AIDS patients with MD ( $p < .0001$ ) and ND ( $p < .001$ ). TrkB specific primers were 5'-CCTGAAGGATGCC AGTGACAA-3' (forward) and 3'-CTCCACGCAGACGCCATA-3' (reverse). (B) Expression of HOHO1 mRNA. HOHO1 levels are 9.4-fold, 5.7-fold, and 6.1-fold increased in the cortex of patients with SD ( $p < .001$ ), MD ( $p = .01$ ), and ND ( $p < .001$ ) as compared to brain tissue of SMCs. HOHO1 specific primers were 5'-GGTGTCTGGGCTACTT GCTCT-3' (forward) and 3'-ATAGCCGACGCTCCACCGAC-3' (reverse). TIFIB-specific quantitative PCR did not confirm down-regulation of the transcript and is not shown. TIFIB-specific primers were 5'-CATATAGCCCGTAAAGCTGTGGAA-3' (forward) and 3'-GCAACACCAACCAATATCTTCAAT-3' (reverse).

confirm alternate transcriptional regulation of TIFIB, which was present at lower levels in cortical tissue of severely demented AIDS patients according to mRNA differential display and reverse Northern analysis. The relatively low number of genes with confirmed differential expression underlines the importance of stringent verification procedures to prove the specificity of results obtained by the mRNA differential display technique.

This may be even more important when assessing gene expression in whole tissue rather than in cell lines cultured under uniform conditions. In our study, drawbacks of the technique were failure of some primer combinations to yield representative patterns of bands and unsuccessful reamplification and sequencing of cDNA fragments resulting from contaminating unrelated DNA sequences as previously described (12,13,18). In addition, the missing detection of weak to moderate alterations in expression of selected genes between test samples and/or the absence of low-level transcripts within the display may account for the low number of identified candidate genes. Furthermore, these technical limitations most likely explain why we did not detect up-regulation of gene transcripts with demonstrated importance in the pathogenesis of AIDS dementia such as TNF- $\alpha$  and inducible nitric oxide synthase (iNOS) (18-21). Advanced technologies, such as cDNA microarray analysis, are promising tools for a more comprehensive assessment of the HIV-1-induced gene expression pattern within the CNS (22).

The most important finding of this study is the marked up-regulation of TrkB mRNA in the cortex of severely demented AIDS patients. Recent evidence suggests that the interaction of Trks with its ligands BDNF and NT-4/5 mediates an extremely potent endogenous neuroexcitatory action (23). BDNF and other neurotrophins are known for their trophic effects and the induction of TrkB expression could merely reflect a compensatory mechanism to prevent progressive neuronal loss. However, abnormal neurotrophic regulation may also affect neuronal survival and promote excitotoxic neuronal injury (24,25). One mediator of neurotrophin-induced excitotoxicity is the modulation of both neuronal and immunologic NO synthase (nNOS, iNOS) activity (26-28). Our previous studies have demonstrated that iNOS is markedly elevated in the cortex of patients with severe AIDS dementia and its induction via the viral glycoprotein gp41 is linked to HIV-1-related cognitive dysfunction (19,21). In the cortex of HIV-1-infected individuals, iNOS expression occurs predominantly in microglial cells (29). Interestingly, in AIDS dementia, but not in Alzheimer's disease, expression of both TrkB and BDNF is induced in reactive astrocytes and activated microglial cells, respectively (30,31). Because BDNF and NT4 promote microglial activation *in vitro* (32,33), continuous high-level coexpression of neurotrophins and TrkB may contribute to the marked stimulation of glial cells and macrophages in HIV-1-infected brains, which is known to affect the degree of cognitive decline (34). This paracrine effect could also potentiate the iNOS induction associated with advanced AIDS dementia that may in turn limit the neuroprotective potential of the TrkB/BDNF/NT-4/5 pathway. The interference of iNOS induction with regenerative mechanisms provides additional

evidence that inhibitors of iNOS could exert therapeutic effects in HIV-1-associated cognitive decline.

HOHO1, the second up-regulated gene, was first isolated from human brain cDNA and is identical in its coding sequence to TWIK1 (anion of P domains in a weak inwardly rectifying K<sup>+</sup> channel), which belongs to a novel structural and functional group of potassium channels (35). In both humans and mice its expression is particularly abundant in brain and heart, and within murine brain high levels are present in cerebellar granule cells, brainstem, hippocampus, and cerebral cortex (36-38). TWIK1 behaves as an inwardly rectifying potassium channel and is thought to be involved in the control of background K<sup>+</sup> membrane conductances (36,37). In our study, levels of HOHO1 mRNA were moderately increased within the cortex of HIV-1-infected patients with peak levels in brain tissue of severely demented individuals. Although the role of HOHO1 mRNA up-regulation in AIDS-related cognitive dysfunction remains speculative, the modification of K<sup>+</sup> currents may occur early following HIV-1 invasion of the CNS and may have consequences for neuronal function through disturbance of local ion homeostasis. Consistent with this hypothesis, several studies have shown that the HIV-1 coat proteins gp160 and gp120 as well as the regulatory protein Nef modify the K<sup>+</sup> conductance of various potassium channels in both lymphocyte and glial cell lines or neurons (39-43). Furthermore, the release of cytokines and other immune mediators affect K<sup>+</sup> currents in astrocytes (44,45) and may additionally impair basic glial and neuronal properties. Further characterization of HIV-1-induced alterations of potassium channel function offers important perspectives for the development of novel pharmacologic strategies against the emergence of AIDS-related cognitive dysfunction.

In conclusion, using mRNA differential display analysis we identified the TrkB/BDNF/NT-4/5 pathway and the inward rectifying potassium channel HOHO-1 as two novel candidate mediators associated with the complex disease process of AIDS dementia. Abnormal neurotrophic regulation may enhance excitotoxic pathomechanisms via sustained activation of microglia and macrophages within the brain of HIV-1-infected individuals and alterations of potassium channel function may affect the pathogenesis of AIDS dementia through interference with membrane excitability of various cell types. Future studies will have to define in more detail the *in vivo* relevance of these findings and their potential pharmacologic implications.

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